INTERNATIONAL STUDY ON Artemia XXXIII. Promising Results in Larval Rearing of Penaeus stylirostris Using a Prepared Diet as Algal Substitute and for Artemia Enrichment

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INTERNATIONAL STUDY ON *Artemia*[1]. XXXIII. PROMISING RESULTS IN LARVAL REARING OF *Penaeus stylirostris* USING A PREPARED DIET AS ALGAL SUBSTITUTE AND FOR *Artemia* ENRICHMENT[2]

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ABSTRACT

The importance of highly unsaturated fatty acids (HUFA) such as 20:5ω3 and 22:6ω3 has been investigated for the larvae of the blue shrimp *Penaeus stylirostris*. HUFA-rich or HUFA-enriched *Artemia* nauplii yielded higher production results in the hatchery than HUFA-poor *Artemia*, especially when yeast was used as a substitute for algae during the early larval stages. Best results were obtained when a HUFA-rich formulated diet was used as a supplement to the yeast and as an enrichment for the *Artemia* nauplii. Differences in production results could be attributed to the HUFA content in living and non-living larval diets.

INTRODUCTION

The commercial availability of different sources of brine shrimp cysts has revealed a large variation in the nutritional quality of *Artemia* nauplii for larvae of several species of crustaceans and fish (Bookhout and Costlow 1970; Wickins 1972; Beck et al. 1980; Johns et al. 1981; Klein-MacPhee et al. 1982; Léger and Sorgeloos 1984; Léger et al. 1985a; Watanabe et al. 1980). Besides variation between *Artemia* strains, differences between batches of the same strain have also been reported (Johns et al. 1980; Léger et al. 1985a,b).

Similar to what Watanabe et al. (1978) and Fujita et al. (1980) have reported for marine fish larvae, we have recently demonstrated that

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the presence of sufficient amounts of essential fatty acids such as 20:5ω3 determine the nutritional quality of *Artemia* nauplii for crustacean larvae (Léger et al. 1985b). Other studies using 20:5ω3-lacking and 20:5ω3-fortified *Artemia* nauplii as food source for the mysid shrimp *Mysidopsis bahia* have clearly confirmed the critical role of highly unsaturated fatty acids in this marine shrimp (Léger, unpublished data).

In order to verify the latter academic findings for a commercial species, a study has been carried out with the blue shrimp *Penaeus stylirostris* during the winter of 1982-83 at the Ralston Purina hatchery Agromarina de Panama S.A. in Panama.

After a series of preliminary experiments aimed at defining an adequate experimental setup, a first experiment was conducted with 20:5ω3-rich, 20:5ω3-poor and 20:5ω3-fortified *Artemia* fed from early mysis until six days after post-larval metamorphosis. In a second experiment we investigated the need for highly unsaturated fatty acids in naupliar and protozoean feeding of *P. stylirostris*. In this experiment we used algae (*Chaetoceros* and *Tetraselmis*) as a HUFA-rich, Fleischmann yeast as a HUFA-lacking and Fleischmann yeast supplemented with a HUFA-enriched product as a HUFA-fortified diet. Fleischmann yeast was used as an adequate algal substitute for *P. stylirostris* (Mock et al. 1980).

The results of these experiments are described in this paper.

MATERIALS AND METHODS

ARTEMIA PREPARATIONS

Based on their HUFA content, two different batches of San Francisco Bay Brand® cysts were selected for this study: i.e., HUFA-rich SFBB 236-2016 and HUFA-poor SFBB 1628 (Léger et al. 1985b). The first batch was harvested from solar saltworks in the San Francisco Bay and the second from the more northern San Pablo Bay in California (USA). Cyst densities of 2 g/l were incubated in one µm filtered natural seawater (30 ppt) at 28°C under continuous illumination and aeration in polyethylene cylindroconical bags. After T_{90}-hours incubation (Vanhaecke and Sorgeloos 1982), freshly hatched Instar I nauplii were separated from hatching debris and thoroughly rinsed with seawater. HUFA-fortified *Artemia* nauplii were produced through 24-hour bioencapsulation of a HUFA-enrichment product (AA18-Artemia Systems N.V., Ghent, Belgium) in the *Artemia* nauplii. The enrichment product consists of a micronized (average particle size 5 µm) dry powder containing 8.5% 20:5ω3 and 9.9% 22:6ω3 of total fatty acids. Freshly hatched *Artemia* nauplii were transferred into a suspension of 0.6 g enrichment product per liter of one µm filtered natural seawater under continuous aeration in a cylindroconical plastic bag at 30°C. After 24 hours the enriched meta-nauplii were harvested on a 120 µm screen and thoroughly rinsed with filtered seawater prior to feeding.

PENAEUS CULTURE TESTS

In Experiment I (see Fig. 1), *P. stylirostris* larvae from one brood stock induced spawn were stocked at the nauplius IV stage (N^{IV}) in nine (3 x 3 replicates) 200 L flatbottom outdoor tanks filled with one µm filtered natural seawater (30 ppt) and provided with moderate airstone aeration at the center and periphery of the tank. Stocking density was

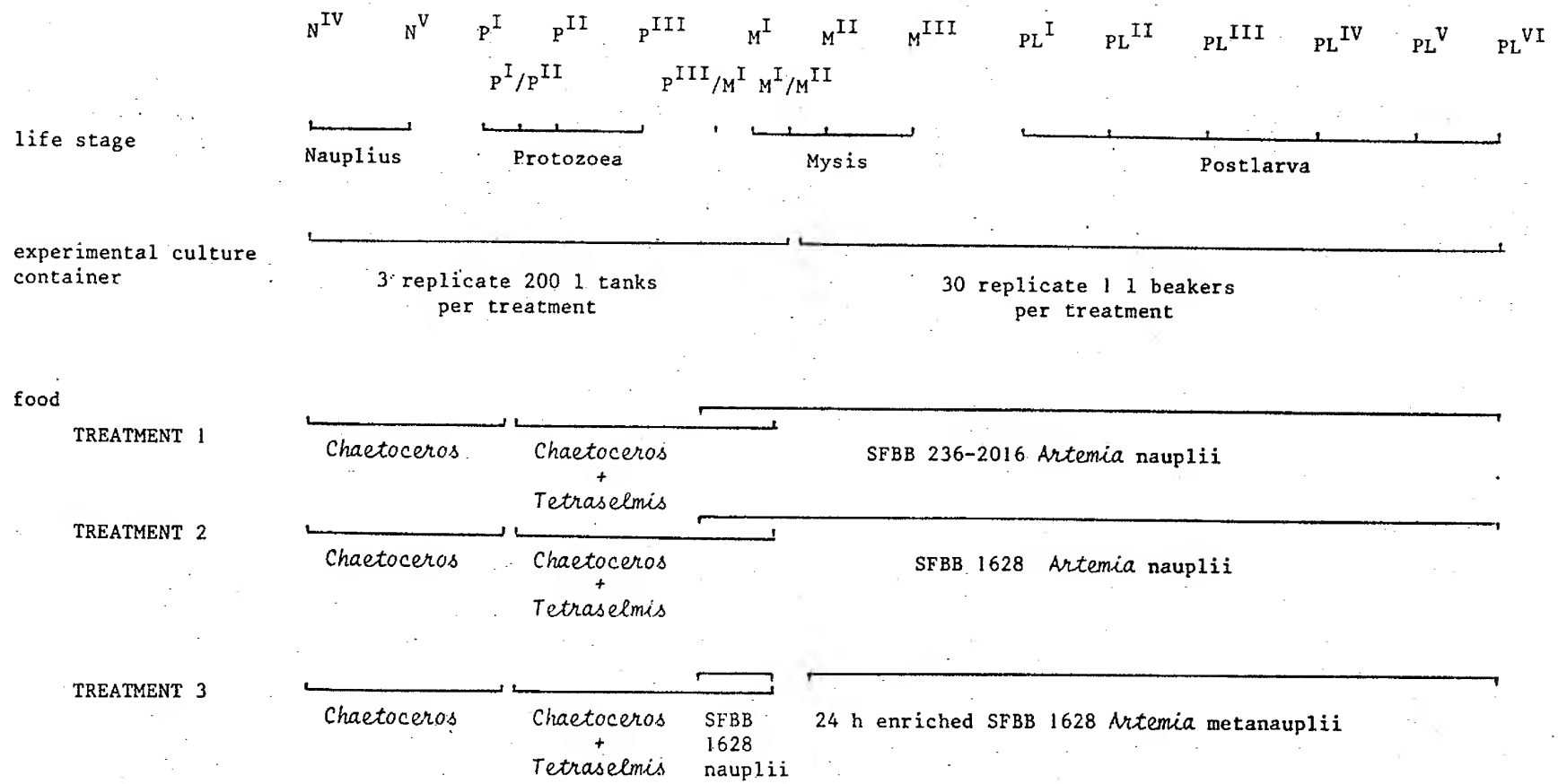

Figure 1. Schematic outline of *P. stylirostris* culture Experiment 1. N = nauplius stage; P = protozoea stage; M = mysis stage; PL = postlarva stage.

40 larvae L^{-1} and temperature in the tanks fluctuated between 28°C and 30°C. No artificial light was used. More or less 80% of the water was renewed every two days. Larvae were fed at first with *Chaetoceros gracilis* and from Protozoea I-II stage (P^{I}/P^{II}) until Mysis I-II stage (M^{I}/M^{II}) with a mixture of *Chaetoceros gracilis* and *Tetraselmis chuii* cultured separately in outdoor conditions. Twice a day cell concentrations were monitored (haematocytometer) and adjusted according to the operational feeding regime. Freshly hatched *Artemia* nauplii were added from P^{III}/M^{I} (3 tanks with SFBB 236-2016, 6 tanks with SFBB 1628). Nauplii concentrations were verified twice a day and adjusted so as to maintain operational levels. At Mysis I-II stage shrimp larvae were transferred in one-liter beakers in one µm filtered and UV-treated natural seawater (30 ppt) without aeration. Thirty beakers per treatment were set up in indoor conditions under continuous illumination at 28°C±0.5°C. Larvae were stocked at 20 individuals per L^{-1} and fed *Artemia* nauplii ad libitum (see Table 1). The treatments consisted of freshly hatched SFBB 236-2016 *Artemia* nauplii (treatment 1), freshly hatched SFBB 1628 (treatment 2) and 24-hour enriched SFBB 1628 meta-nauplii (treatment 3).

Table 1. Levels of *Artemia* Nauplii Fed in the one-liter Beakers for *P. stylirostris* Larvae from Mysis I-II Stage (M^{I}/M^{II}) Through Postlarva VI Stage (PL^{VI}) Assuring ad libitum Feeding. Time of water exchange is also given.

	Larval stage								
	M^{I}	M^{II}	M^{III}	PL^{I}	PL^{II}	PL^{III}	PL^{IV}	PL^{V}	PL^{VI}
Artemia nauplii ml^{-1}	2	4	5	5	8	5	8.5	5	8
Water exchange	-	-	+	-	+	-	+	-	+

Water was changed every two days by siphoning, taking care not to damage the animals. Excess *Artemia* and dead larvae were removed daily prior to feeding. Mortality in the one-liter beakers was recorded daily and survival was calculated at the end of the experiment (Postlarva VI stage). Individual wet weight of all surviving postlarvae (excess water removal on absorbent papercloth) was measured with an analytical balance (accuracy 0.1 mg). Biomass production was calculated as the product of survival and individual wet weight.

For Experiment 2 (see Fig. 2), the same experimental setup was used as in the first. Seven treatments of three replicate 200 L tanks were run. Two treatments were fed algae as described in the first experiment, and five others were fed Fleischmann yeast type 7B (Standard Brand Foods, New York, N.Y.): i.e., two treatments received yeast only, three treatments were fed with a 1:1 mixture of Fleischmann yeast plus *Artemia* HUFA-enrichment diet. Cell concentrations were kept at 50,000 cells ml^{-1} (Mock et al. 1980). A suspension of Fleischmann yeast in filtered seawater was prepared daily with a magnetic stirrer, whereas the enrichment product was homogenized in seawater using a kitchen blender. From Protozoea III to Mysis I stage freshly hatched Instar I nauplii from SFBB

236-2016 *Artemia* cysts were fed at operational concentrations in treatments 1, 3 and 5 (see Fig. 2). SFBB 1628 nauplii were fed in treatments 2, 4 and 6, and 24-hour HUFA-enriched meta-nauplii from SFBB 1628 *Artemia* cysts in treatment 7. These treatments were continued after transferring the shrimp larvae at Mysis I-II stage in 15 replicate one-liter beakers per treatment. Prior to transfer larval survival was assessed by subsampling. From this stage only *Artemia* was fed at concentrations given in Table 1. After metamorphosis to post-larval stage overall survival was calculated. Developmental rate was recorded throughout the experiment.

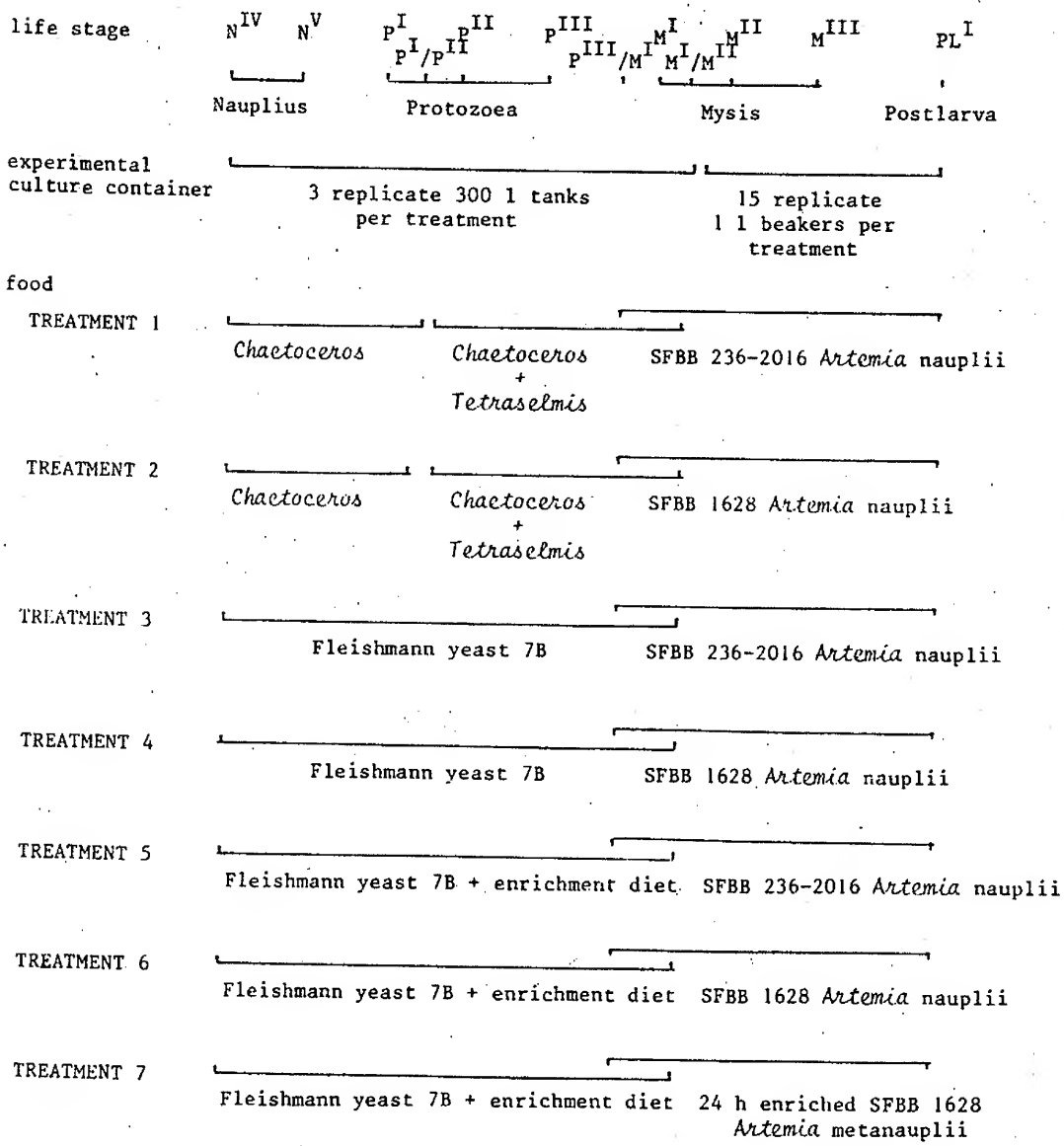

Figure 2. Schematic outline of *P. stylirostris* culture Experiment 2. N = nauplius stage; P = protozoea stage; M = mysis stage; PL = postlarva stage.

DATA ANALYSIS

Data were treated statistically in a one-way analysis of variance. Prior to analysis, survival data were normalized by transformation to angles (arcsin $\sqrt{\%}$) (Snedecor and Cochran 1967). Duncan's multiple range test was used to determine significant differences among means (Goodnight 1979).

FATTY ACID ANALYSIS

Fatty acid profile was determined on freshly hatched and enriched *Artemia* nauplii as well as on Fleischmann yeast type 7B and the HUFA-enrichment diet. After homogenization with an ultrasonic homogenizer (Sonifier B12, Branson Sonic Power Company, Connecticut, USA), lipid extraction, saponification and esterification were done according to the procedure described in Schauer and Simpson (1978). Fatty acid methyl esters were injected on a capillary column (25 m Fused Silica, I.D. 0.32 mm; liquid phase Silar 10C, film thickness 0.3 µm) installed in a Carlo Erba Fractovap 2330 gas chromatograph. Operating conditions were as follows: solid injector, carrier gas--hydrogen, flow rate--1.9 ml·min; F.I.D.-detection, oven temperature program--154 to 200°C at 1.5°C min^{-1}. Peak identification and quantification was done with a calibrated plotter-integrator (Hewlett Packard 3390A). The internal standard procedure using 20:2ω6 as internal standard was used for quantitative analyses.

RESULTS

In the first experiment significant differences (α:0.05) in survival and biomass production are noticed in larval *Penaeus stylirostris* fed with either SFBB 236-2016 or SFBB 1628 *Artemia* nauplii (see Table 2 and Fig. 3). After HUFA-enrichment inferior SFBB 1628 *Artemia* guarantee equal survival and significant (α:0.05) growth in the shrimp larvae as compared to SFBB 236-2016. In the second experiment (see Table 3), when raising Nauplius IV until Mysis I-II stage, only the diet combination Fleischmann yeast followed by SFBB 1628 *Artemia* nauplii (treatment 4) results in a significantly inferior survival as compared to the other diet combinations. More pronounced differences are noticed when the experiment is continued until after post-larval metamorphosis. A similar nutritional deficiency is noticed in SFBB 1628 *Artemia* nauplii as in the first experiment (treatment 2). This deficiency is even more accentuated when Fleischmann yeast is used as algal substitute (treatment 4). However, when this yeast is used in combination with the HUFA-enrichment diet no significant difference in survival is notice between the SFBB 236-2016 and SFBB 1628 fed treatments (treatments 5 and 6). The most effective feeding regime appears to be the one using yeast plus enrichment diet and enriched *Artemia* nauplii (treatment 7).

From Table 3 and Figure 4 we further learn that the use of algal substitutes results in a developmental lag of more or less 1.5 to 2.5 days at M^I/M^{II} and 2 to 3 days at PL^I; apparently developmental rates are most affected at Protozoea II and III stage.

Fatty acid analyses (see Table 4) reveal a clear difference in profile between SFBB 236-2016 and SFBB 1628 *Artemia* nauplii, i.e., the former have considerably higher contents of 16:1 (ω7 and other monoënes), 18:1ω7/ω9 and 20:5ω3, and lower levels of 16:2, 18:2ω6, 18:3ω3 and 18:4ω3. Some 22:6ω3 has been identified in SFBB 236-2016. Supplementa-

tion of the SFBB 1628 *Artemia* nauplii with the HUFA-enrichment diet results in a pronounced increase of the fatty acid 20:5ω3 as well as in the appearance of significant levels of 22:6ω3. Fleischmann yeast 7B does not contain any fatty acids higher than 20:1.

Table 2. Survival and Individual Wet Weight of *P. stylirostris* Postlarvae (PL^{VI}) Fed Algae and SFBB 236-2016 *Artemia* Nauplii (Treatment 1), Algae and SFBB 1628 *Artemia* Nauplii (Treatment 2) or Algae and 24 h Enriched SFBB 1628 *Artemia* Nauplii (Treatment 3)

	Treatment		
	1	2	3
	SFBB 236-2016	SFBB 1628	SFBB 1628 24 h enriched
Survival (%)	47.5^{a}	34.0^{b}	45.7^{a}
s	5.2	10.9	5.8
Individual wet wt (mg)	1.8^{b}	1.7^{b}	2.0^{a}
s	0.3	0.3	0.4

^{a,b}Means with different superscript are significantly different (α:0.05).

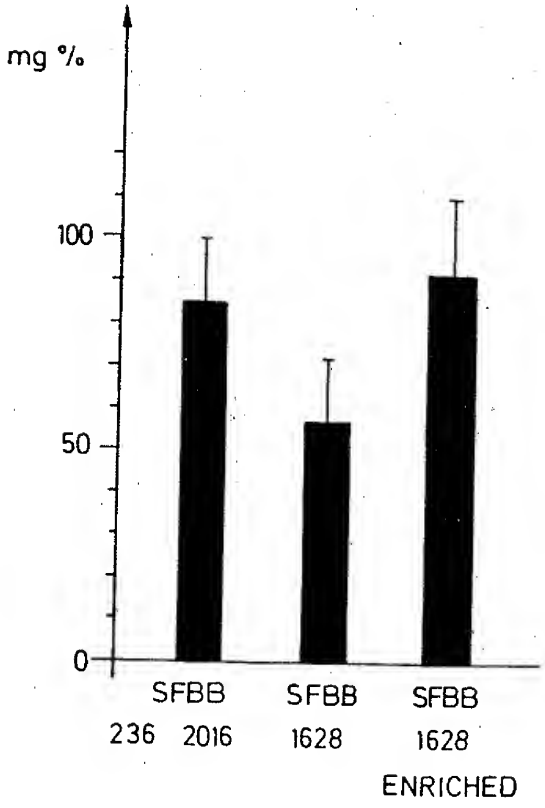

Figure 3. Biomass production of 100 Nauplius IV *P. stylirostris* larvae at Postlarva VI stage in culture Experiment 1 (expressed as mg % wet weight increase).

Table 3. Survival and Developmental Rate of *P. stylirostris* at Mysis I-II Stage (M^I/M^{II}) and First Post-larval Stage (PL^I) Fed Algae and SFBB 236-2016 resp. SFBB 1628 *Artemia* Nauplii (Treatment 1 resp. 2), Fleischmann Yeast and SFBB 236-2016 resp. SFBB 1628 *Artemia* Nauplii (Treatment 3 resp. 4), and Fleischmann Yeast plus Enrichment Diet and SFBB 236-2016, SFBB 1628 resp. 24 h Enriched SFBB 1628 *Artemia* (Treatment 5, 6 resp. 7)

	Treatment						
	1	2	3	4	5	6	7
	Algae		Yeast		Yeast + enrichment diet		
	SFBB 236-2016	SFBB 1628	SFBB 236-2016	SFBB 1628	SFBB 236-2016	SFBB 1628	SFBB 1628 enriched
$N^{IV} \rightarrow M^I/M^{II}$							
Survival (%)	88.3^a	78.8^{ab}	79.3^{ab}	72.2^b	83.3^{ab}	90.8^a	91.8^a
s	8.3	15.7	2.1	19.5	9.2	3.3	9.6
Developmental rate (days)	5.5 ± 0	5.5 ± 0	7 ± 0.5	7 ± 0.5	8 ± 0.5	8 ± 0.5	8 ± 0.5
$N^{IV} \rightarrow PL^I$							
Survival (%)	73.6^{ab}	49.4^c	72.7^{ab}	16.7^d	66.9^b	77.5^{ab}	83.8^a
s	10.9	9.9	7.2	8.8	9.4	6.8	9.1
Developmental rate (days)	7.5 ± 0	7.5 ± 0	9.5 ± 0.5	9.5 ± 0.5	10.5 ± 0.5	10.5 ± 0.5	10.5 ± 0.5

^{a,b,c,d}Means with different superscript are significantly different (α:0.05).

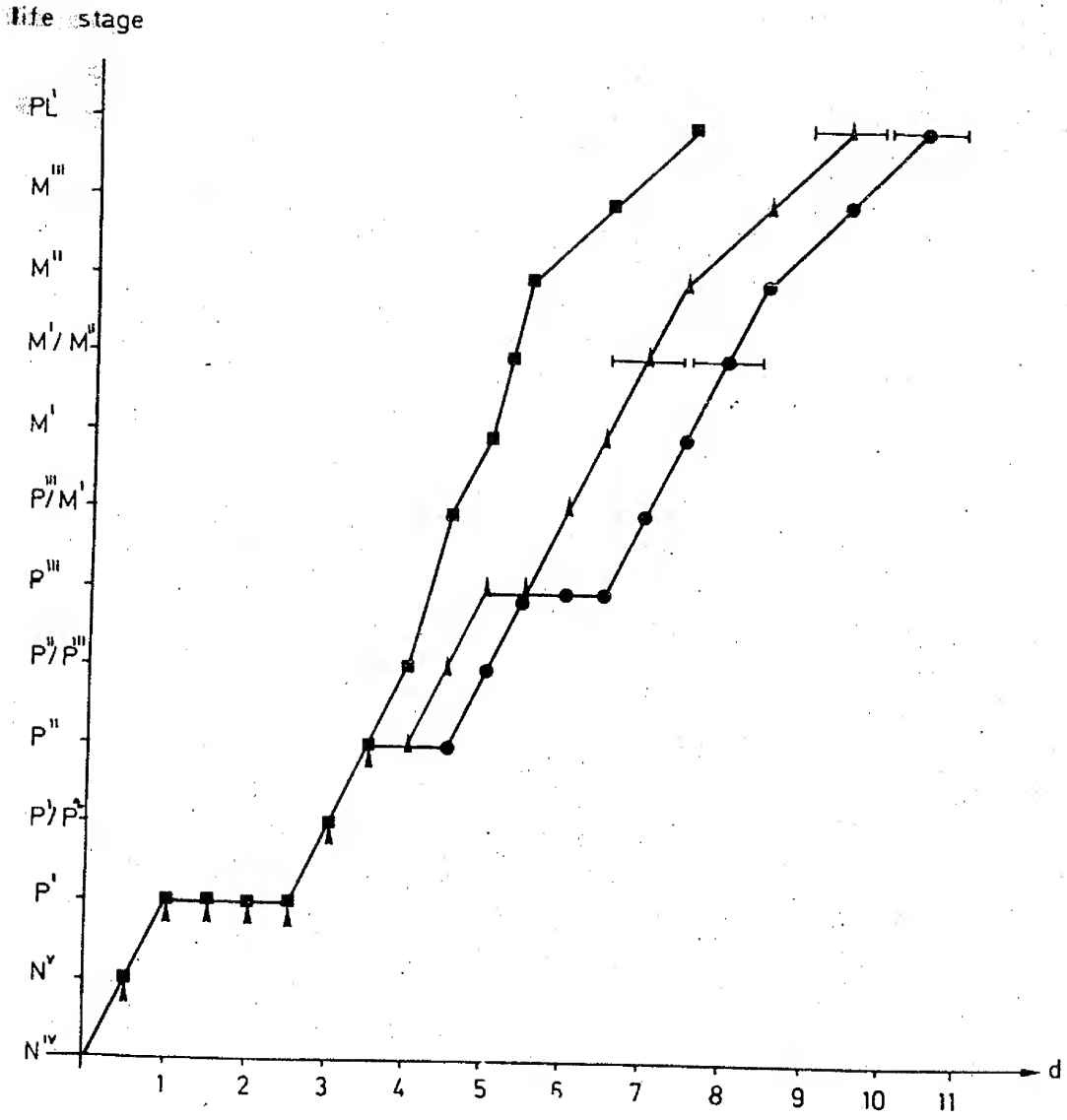

Figure 4. Developmental rate of *P. stylirostris* larvae fed algae (■), Fleischmann yeast (▲) and Fleischmann yeast plus HUFA-enrichment diet (●). N = nauplius stage; P = protozoea stage; M = mysis stage; PL = postlarva stage.

CONCLUSIONS

Similar to what has been reported for many other predator larvae (see review in Léger et al. 1985a) the use of different *Artemia* strains (e.g. San Francisco Bay and San Pablo Bay) can markedly affect the culture success of larval *P. stylirostris*. The results obtained in this study with *P. stylirostris* using SFBB 236-2016, containing relatively high levels of 20:5ω3, and SFBB 1628, containing low levels of 20:5ω3, confirm earlier findings that the presence of sufficient amounts of highly unsaturated fatty acids determines the nutritional effectiveness of *Artemia* as a larval food source in marine crustaceans (Léger et al. 1985a).

Table 4. Fatty Acid Profile of (1) Fleischmann Yeast Type 7B, (2) Enrichment Diet AA18, (3) Freshly Hatched SFBB 236-2016 *Artemia* Nauplii, (4) Freshly Hatched SFBB 1628 *Artemia* Nauplii, and (5) 24 h Enriched SFBB 1628 *Artemia* Nauplii. Concentrations are expressed in (A) percent fatty acid methyl ester of total fatty acid methyl esters and (B) mg fatty acid methyl ester per gram dry weight.

FAME[a]	1		2		3		4		5	
	A	B	A	B	A	B	A	B	A	B
14:0	0.50	0.06	3.40	2.54	1.73	2.20	0.90	0.93	1.60	2.20
14:1	0.14	0.02	0.20	0.13	1.02	1.30	1.50	1.55	0.80	1.00
14:2	0.37	0.05	0.10	0.10	-[b]	-	0.40	0.42	0.20	0.23
15:0	0.51	0.06	0.40	0.30	0.44	0.56	0.40	0.38	0.30	0.40
15:1	-	-	0.10	0.10	0.23	0.30	0.75	0.75	0.50	0.60
16:0	12.15	1.52	12.50	9.50	12.50	15.90	10.00	10.40	11.30	15.20
16:1ω9	35.00	4.38	0.50	0.40	0.20	0.25	0.74	0.80	0.94	1.30
16:1ω7			7.40	5.60	20.85	26.53	4.94	5.20	7.00	9.40
16:2	0.30	0.04	0.20	0.15	0.30	0.34	0.20	0.20	0.10	0.10
16:3	0.94	0.12	0.34	0.30	0.74	0.94	1.60	1.62	0.10	0.10
17:0	0.74	0.10	0.30	0.20	-	-	0.50	0.50	0.50	0.70
17:1	-	-	0.43	0.32	-	-	-	-	1.10	1.50
18:0	8.10	1.01	2.90	2.20	3.00	3.80	3.50	3.70	5.50	7.40
18:1ω9	36.30	4.54	19.40	14.70	34.90	44.43	26.20	27.30	24.90	33.40
18:1ω7			3.30	2.50						
18:2ω6	3.50	0.43	1.70	1.30	3.00	3.82	6.40	6.70	4.20	5.70
18:3ω6	-	-	0.10	0.10	0.20	0.27	0.50	0.50	0.30	0.36
18:3ω3	0.70	0.10	0.10	0.05	7.00	8.87	28.55	29.70	15.40	20.60
18:4ω3	-	-	1.80	1.35	1.30	1.61	6.10	6.40	2.80	3.80
19:0	-	-	0.04	0.04	-	-	0.30	0.34	0.40	0.50
19:4?	-	-	0.13	0.10	-	-	-	-	0.03	0.04
20:0	0.30	0.04	0.10	0.10	0.20	0.27	0.20	0.24	0.30	0.36
20:1ω9/ω7	0.30	0.04	12.94	9.80	-	-	1.25	1.30	5.90	7.90
20:2ω6	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace
20:3ω6	-	-	0.10	0.05	-	-	-	-	-	-
20:3ω3	-	-	0.14	0.10	0.40	0.52	0.30	0.30	-	-
20:4ω6	-	-	0.10	0.05	-	-	0.20	0.20	-	-
20:4ω3	-	-	0.50	0.34	-	-	1.40	1.50	1.00	1.35
20:5ω3	-	-	8.50	6.50	8.84	11.24	0.50	0.50	5.90	7.90
21:5	-	-	0.60	0.50	trace	trace	1.52	1.60	1.00	1.34
22:1	-	-	8.50	6.40	0.40	0.52	trace	trace	2.80	3.80
22:3ω3	-	-	0.50	0.36	-	-	-	-	-	-
22:4ω6	-	-	0.50	0.38	-	-	-	-	0.15	0.20
22:4ω3	-	-	0.26	0.20	-	-	0.30	0.30	0.30	0.43
22:5ω6	-	-	0.20	0.14	-	-	-	-	-	-
22:5ω3	-	-	1.00	0.80	-	-	-	-	0.60	0.82
22:6ω3	-	-	9.90	7.50	0.65	0.83	-	-	3.42	4.60
24:0	-	-	0.20	0.16	-	-	0.20	0.20	0.14	0.20
n.i.p.[c]	-	-	0.45	0.37	-	-	0.70	0.75	0.60	0.70
ΣHUFAω3[d]	0	0	20.80	15.90	9.89	13.11	2.70	2.80	11.22	15.10

[a]Fatty Acid Methyl Ester. [b]Not detected. [c]Non-identified peaks.

[d]Sum of ω3 Fatty Acid Methyl Esters > C20.

This is further confirmed by the fact that the HUFA-enriched SFBB 1628 *Artemia* nauplii are significantly more effective as food for *P. stylirostris* than the freshly hatched nauplii of the same strain. The better growth in HUFA-enriched SFBB 1628 *Artemia* fed larvae as compared to the SFBB 236-2016 *Artemia* fed larvae could be attributed to the presence of higher amounts of the HUFA 22:6ω3. Further evidence for the essential role of HUFA's in larval shrimp nutrition are provided when using Fleischmann yeast 7B (lacking HUFA's) as a substitute for *Chaetoceros gracilis* and *Tetraselmis chuii* (both high in 20:5ω3, Aujero et al. 1983; Chuecas and Riley 1969; Ackman and Tocher 1968): i.e., when feeding 20:5ω3-poor SFBB 1628 *Artemia* inferior results are obtained when larvae have previously been fed on yeast as compared to algae. Since this difference is most pronounced after post-larval metamorphosis, HUFA requirements (especially 20:5ω3) in *P. stylirostris* culture appear to be highest at post-larval metamorphosis. No significant difference is detected between SFBB 236-2016 and SFBB 1628 *Artemia* nauplii as larval food for *P. stylirostris* when the algal substitute consists of Fleischmann yeast 7B in combination with the HUFA-enrichment diet. This suggests that HUFA supplementation in early larval stages reduces the critical need for HUFA-rich diets in later larval stages; as a result differences between *Artemia* strains in terms of HUFA contents are also less pronounced.

Since the best results in the *P. stylirostris* culture tests were obtained with the combined use of the HUFA diet as supplement for the yeast and enrichment of *Artemia*, the HUFA formulation appears to supply qualitatively and/or quantitatively more components than present in SFBB 236-2016 and the algae used. This true for the HUFA 22:6ω3 which should allow us to assume that 22:6ω3 might be an essential fatty acid in the larval nutrition of *P. stylirostris*. With 22:6ω3 nearly absent and 20:5ω3 present at inconsistent levels in *Artemia* cysts (Léger et al. 1985b; Schauer et al. 1980; Seidel et al. 1982; Vos et al. 1984; Watanabe et al. 1978), HUFA enrichment may become common practice in larval rearing of *P. stylirostris* and species with similar requirements.

A marked difference in survival between the first and second experiments is observed. Since mortality in larval development of *P. stylirostris* peaks at post-larval metamorphosis and stabilizes thereafter, this difference is not to be attributed to the longer culture period in the first experiment. Differences in offspring quality or the fact that the first experiment was run during the changing of seasons and the second experiment at the start of the dry season, may not be excluded. Indeed, it is known by shrimp farmers in the tropics that changing of seasons adversely affects hatchery production; furthermore, it has been demonstrated that changes in environmental conditions (occurring during the transition of seasons?) can affect the biochemical composition of algae, especially their fatty acid profile (Dickson et al. 1969; Enright 1984). Unfortunately, we were not able to verify this in the present study. Further studies, monitoring fatty acid profiles in phytoplankton and using HUFA-enrichment products in combination with algae could be more elucidating.

Although the use of the HUFA diet as a supplement for the yeast and/or enrichment for the *Artemia* nauplii does not create secondary problems or inconveniences in hatchery operations, the immediate application as an algal substitute is not to be recommended. Larval developmental rate, especially in the protozoea stages, is delayed by 2 to 3 days when Fleischmann yeast eventually in combination with the enrich-

ment product is used as algal substitute. Any production delay can be translated in less efficient and less rentable operation. Since developmental rate normalizes after feeding only *Artemia* nauplii, this lag appears to be caused by the use of algal substitutes. Non-optimal feeding regimes and subsequent water quality problems may be responsible.

Further work is needed to establish optimal feeding regimes with algal substitutes, the application of which nevertheless seems to be very promising.

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